## REMARKS

In response to the Office Action of June 1, 2004, claim 13 is hereby amended and new claims 27-29 are added. Claims 13, 15-16, 19, 23 and 26 were rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement, under 35 U.S.C. § 102(b) as being anticipated by Boulware *et al.* (1981) J. of Natural Products 44(2):200-205, as further evidenced by Southard *et al.*, U.S. Pat. No. 5,013,553 and under 35 U.S.C. § 103(a) as being unpatentable over Boulware *et al.* (1981) J. of Natural Products 44(2):200-205. Each of these rejections is discussed below.

Rejections under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 13, 15-16, 19, 23 and 26 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The Examiner maintains that the "claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." To satisfy the written description requirement, the written description must clearly allow persons of ordinary skill in the art to recognize that the inventor invented what is claimed. In re Gosteli, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989). Specifically, the Examiner asserts that the phrase "single solvent system" cannot be found in the Specification as filed and thus is deemed to be new matter.

Claim 13 is drawn to a method for the isolation and purification of an aporphine alkaloid comprising the steps of extracting the ground biomass of a plant containing aporphine alkaloids with a solvent; neutralizing and concentrating the extract and purifying by selected chromatographic methods. In response to this rejection, claim 13 has been amended to read "performing a single exhaustive extraction" of a ground biomass of a plant containing aporphine alkaloids with a "hydroxylated polar" solvent "or mixture of hydroxylated polar solvents." Support for this amendment can be found in Example 2 which provides that "Southern Prickly Ash bark powder . . . was extracted exhaustively with hot methanol (Soxhlet extraction) for 48 hours" (Specification, page 15, lines 11-12) and on page 11 of the Specification, which provides

that the solvent or mixtures thereof that can be used for extraction includes "acidified water, an acidified water miscible **hydroxylated** organic solvent including . . . methanol or ethanol and an acidified mixture of alcohol or other water miscible **hydroxylated** organic solvent and water." (Specification, page 11, lines 18-22, emphasis added). Further support for this amendment can be found on page 12 of the Specification, which provides that "isoquinoline alkaloids can be extracted from plants with deionized (DI) water, acidic aqueous solutions or high polarity solvents." (Specification, page 12, lines 5-6). Applicant maintains that claim 13, as amended, overcomes this rejection and respectfully requests that this rejection be withdrawn. Additionally, new claims 27-29 have been added which set forth the preferred solvents and mixtures thereof for the extraction. Support for new claims 27-29 can be found in the Specification (page 11, lines 18-21, page 12, lines 5-9).

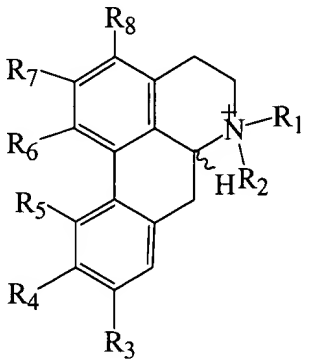
Rejection under 35 U.S.C. § 102(b)

The Court of Appeals for the Federal Circuit has stated that anticipation requires the presence in a single prior art reference of each and every element of the claimed invention. Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co., 730 F.2d 1452, 1458 (Fed. Cir. 1984); Alco Standard Corp. v. Tennessee Valley Auth., 1 USPQ2d 1337, 1341 (Fed. Cir. 1986). "There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention." Scripps Clinic v. Genentech Inc., 18 USPQ2d 1001, 1010 (Fed. Cir. 1991) (citations omitted).

The Examiner has rejected claims 13, 15-16, 23 and 26 under 35 U.S.C. § 102(b) as being anticipated by Boulware *et al.* (1981) J. of Natural Products 44(2):200-205 as further evidenced by Southard *et al.*, U.S. Pat. No. 5,013,553. Briefly, the Examiner reasons that Boulware *et al.* teach that magnoflorine is inherent to species of Zanthoxylum. The Examiner further provides that Boulware *et al.* specifically outline an extraction protocol which includes: extraction with hexane followed by methanol, dissolving the methanol residue in 1 M sulfuric acid, extraction with chloroform, neutralization to pH 6, extraction with butanol followed by concentration, and purification via silica gel. The Examiner concludes that the reference teaches

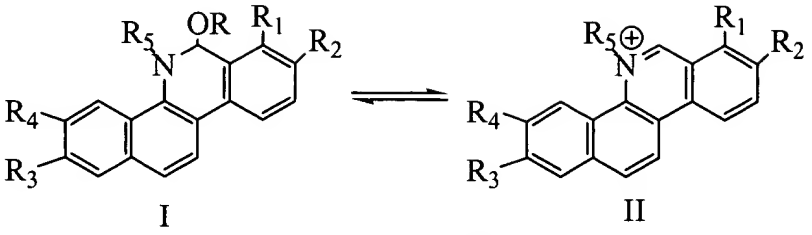
every method step recited in the instant claims, and thus anticipates the claimed invention. The Examiner reiterates that silica gel is a type of ion exchange column and therefore satisfies the chromatographic method of step (c). The Examiner further provides that Boulware *et al.* used a "single solvent system" in that they used one system comprising solvents in order to purify the Zanthoxylum extract. The Examiner makes no mention of Southard *et al.*

The present invention is drawn to a method for the isolation and purification of a specific class of aporphine alkaloids having the following structure:




The method as set forth in claim 13, as amended, is comprised of the steps of consisting of: (a) performing a single exhaustive extraction of a ground biomass of a plant containing aporphine alkaloids with a hydroxylated polar solvent or mixture of hydroxylated polar solvents, wherein said solvent or mixture thereof is optionally acidified; (b) optional neutralization of said acidified solvent and concentration of the neutralized extract; and (c) purification of said extract by a column chromatographic method, wherein said chromatographic method is selected from the group consisting of ion exchange chromatography, reverse phase chromatography, size exclusion chromatography, ultra-filtration or a combination of two or more of these methods.

Southard *et al.* describe a method for isolating benzophenanthridine alkaloids, which have the following structures:



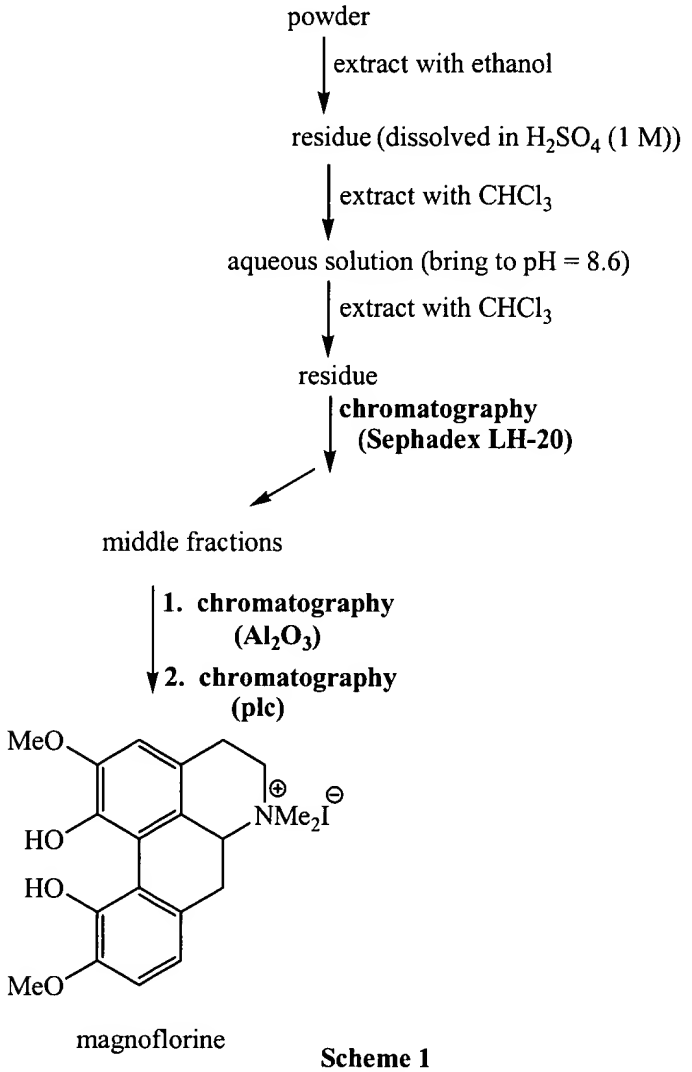

I II

benzophenanthridine alkaloids

from plants via extraction with a mineral acid/alcohol solvent, precipitation with a base and dissolution in water, precipitation and drying and further purification by silica gel column chromatography. These compounds exist in the form of the iminium ion (structure II) only at very low pH. Southard *et al.* do not disclose or suggest that their method can be extended to aporphine alkaloids. As noted above, anticipation requires the presence in a single prior art reference of each and every element of the claimed invention. The present invention, as amended, describes and claims a method for the isolation and purification of a specific group of aporphine alkaloids. These alkaloids contain a positively charged quaternary amine group which differentiates the physical properties of the claimed compounds from the compounds isolated and purified in the Southard *et al.* reference. Applicant maintains that the claims, as amended, are not anticipated by the Southard *et al.* reference and therefore respectfully requests that this rejection be withdrawn.

Boulware *et al.* teach the separation and purification of a number of alkaloids from *Z. microcarpum* and *Z. procerum* using various methods. According to one method, used in the isolation and purification of magnoflorine, the debarked wood of *Z. microcarpum* is ground to a powder, extracted and purified by chromatography as illustrated in Scheme 1, below. With reference to Scheme 1, following extraction with ethanol and chloroform the residue is first subjected to chromatography on a Sephadex LH-20 column. Sephadex LH-20 is a gel used in size exclusion chromatography. The middle fractions from the Sephadex LH-20 column were then further purified via chromatography on $Al_2O_3$, which is used in adsorption chromatography, followed by final purification by preparative layer chromatography (plc) to provide magnoflorine. It cannot be determined from the reference what stationary phase was used on the

PLC plates, however it is most likely silica gel since silica was used in the method set forth in Scheme 2, below. PLC chromatography is used for very small scale (mg quantity) separations. PLC plates are typically coated with the same silica gel used for analytical TLC plates. The method outlined in Scheme 1 includes three extractions and three chromatography steps, two of which are adsorption chromatography.

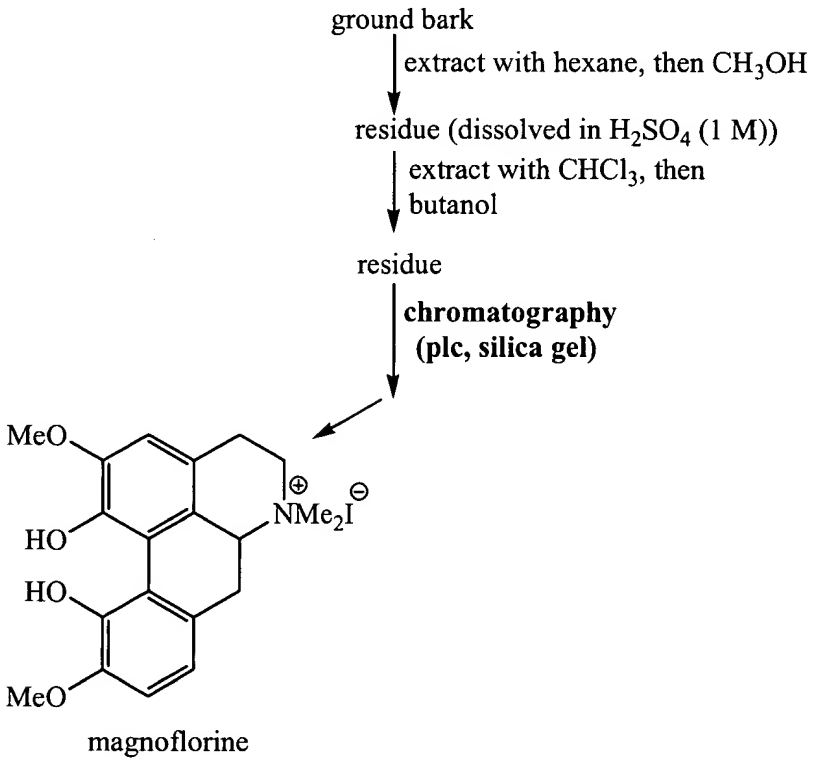



magnoflorine

**Scheme 1**

Scheme 2 outlines a second method employed by Boulware *et al.* for the isolation and purification of magnoflorine, using adsorption phase chromatography (PLC on silica gel). The

method outlined in Scheme 2 involves four extractions (hexane, methanol, chloroform and butanol), followed by preparative layer chromatography on silica gel.




magnoflorine

**Scheme 2**

As noted above, claim 13 of the instant invention, as amended, is drawn to a method for the isolation and purification of an aporphine alkaloid comprising the steps of performing a single exhaustive extraction on the ground biomass of a plant containing aporphine alkaloids with a hydroxylated polar solvent or mixture of hydroxylated polar solvents; neutralizing and concentrating the extract and purifying by selected column chromatographic methods. In the Amendment and remarks document filed on August 6, 2003, claim 13 was amended to read that the method "consists of" rather than "comprises" to exclude any additional steps required by the Boulware *et al.* reference. Thus, as amended, claim 13 is drawn to a method consisting of a single extraction with a hydroxylated polar solvent and purification by column chromatography.

The first method disclosed by Boulware *et al.* (outlined in Scheme 1) includes three extractions (ethanol, chloroform and chloroform) and three chromatography steps, two of which

are adsorption chromatography with the final chromatography being performed on a preparative plate. As noted previously, preparative layer chromatography is performed on a very small (mg) scale and is typically used for difficult separations. Preparative layer chromatography is not a commercially viable method to obtain large (g) scale amounts of product. Examples 3 and 4 of the instant application, on the other hand, disclose a commercially viable method for the large scale isolation and purification of aporphine alkaloids. The method involves a single extraction with a hydroxylated polar solvent, followed by purification by column chromatography. With reference to Example 4, approximately 2 grams of product were obtained following purification. (Specification, page 18, lines 24-25). Applicant maintains that, as amended, claim 13 is not anticipated by the method of Boulware *et al.* as outlined in Scheme 1.

The second method disclosed by Boulware *et al.* (outlined in Scheme 2) includes four extractions (hexane, methanol, chloroform and butanol), followed by preparative layer chromatography on silica gel. For the reasons discussed above with respect to the first method Applicant maintains that, as amended, claim 13 is not anticipated by the method of Boulware *et al.* as outlined in Scheme 2.

With respect to the Examiner's assertion that silica gel is a type of ion exchange column Applicant reiterates for the reasons discussed in detail below that as would be understood by one of skill in the art silica gel chromatography is not a type of ion exchange chromatography.

Liquid column chromatography is used to separate organic compounds on the basis of their charge, size, shape and solubility. A chromatography consists of a mobile phase (solvent and molecules to be separated) and a stationary phase (paper, resin, beads or membranes) through which the mobile phase travels. Molecules travel through the stationary phase at different rates depending on their chemistry and the type of chromatography being employed. There are several distinct types of chromatography including: adsorption, partition, ion exchange and size exclusion. Included with this document is a copy of a section of a chromatography supply catalog entitled "Chromatography Columns and Supplies Catalog" (2003-2004) pp.94-98, obtained from www.waters.com, which describes several different types of chromatography columns in detail.

Normal phase adsorption chromatography is the classical form of chromatography using polar stationary phases and non-polar mobile phases. Adsorption chromatography is based on interactions between the solute and fixed active sites on the stationary phase. The stationary phase is a solid adsorbent, typically unbonded silica gel (Si-OH) or alumina ($Al_2O_3$), packed in a column, spread on a plate or on a porous paper. The mobile phase is usually a liquid solvent. The active sites of the stationary phase interact with the functional groups of the compounds to be separated by noncovalent, nonpolar interactions, Van der Waals forces and hydrophobic interactions. An example is the separation of alcohols from hydrocarbons using silica gel, in which the silanol groups on the silica gel interact with the polar functional groups on the alcohol and retard their progress, resulting in the hydrocarbons eluting first.

In reverse-phase partition chromatography, the stationary phase is non-polar and the mobile phase is polar. Typical mobile phases are mixtures of water or aqueous buffer with methanol, acetonitrile or tetrahydrofuran. Typical stationary phases are silica based bonded phases with aliphatic hydrocarbons (R) as ligands (Si-OR). The most common bonded phases are n-octyldecyl (C18) and n-decyl (C8) chains and phenyl groups. Other packings for reverse-phase chromatography are graphitized carbon and styrene-divinylbenzene packings. Several examples of reverse-phase packings are illustrated on page 95 of the enclosed catalog section.

Ion exchange chromatography allows the separation of ions and polar molecules based on the electrostatic charge of the compounds to be separated. The separation of solutes by their charge is possible using phases that contain fixed ionic charges. Thus, the stationary phase is a resin or gel matrix, which contains covalently bound positive functional groups, such as-$NHR_2^+$ and -$NR_3^+$ (anion exchange column) or negative functional groups, such as $-SO_3^-$, -$OPO_3^-$ and – $COO^-$ (cation exchange column). In the case of silica-based ion-exchangers, the ionic species are attached to the surface using standard silanization techniques. The mobile phase is typically a buffered aqueous solution, which carries a counter-ion whose charge is opposite and in equilibrium with the total charge of the resin. The analytes are eluted using a buffer with a higher ionic strength or different pH in order to weaken the electrostatic interactions between the analytes and the exchangers.

Size exclusion chromatography (also referred to as gel filtration, gel-permeation and molecular sieve chromatography), allows the separation of molecules according to their size or molecular weight. The stationary phase is typically a chemically inert material, such as a gel or a porous inorganic solid. Examples include polyacrylamide polymers, porous glass or porous silica beads. The degree of retention is dependant on the size of the solvated solute molecule relative to the size of the pore. Molecules that are smaller than the holes in the beads get hung up in the beads. Therefore, smaller molecules move through the column more slowly than larger molecules.

Contrary to the Examiner's assertion, absorption phase chromatography, typically performed on nonbonded silica gel containing Si-OH functional groups, is quite distinct from ion-exchange chromatography, which is performed on a stationary phase that contains fixed ionic charges. This fact is well documented and would be well known to those skilled in the art. Furthermore, the Examiner has provided no evidence to the contrary.

Rejection under 35 U.S.C. § 103(a)

The Examiner has rejected claims 13, 15-16, 19, 23 and 26 under 35 U.S.C. § 103(a) as being unpatentable over Boulware *et al.* (1981) J. of Natural Products 44(2):200-205. The Examiner reasons that one of ordinary skill in the art would have been motivated to have purified magnoflorine via a column extractor with silica gel in order to achieve an optimized yield of product on a large scale.

The Examiner bears the burden of establishing a prima facie case of obviousness. In determining obviousness, one must focus on Applicant's invention as a whole. Symbol Technologies Inc. v. Opticon Inc., 19 USPQ2d 1241, 1246 (Fed. Cir. 1991). The primary inquiry is:

whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have had a reasonable likelihood of success. . . . Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure. In re Dow Chemical, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988).

In response to this rejection, Boulware *et al.* neither disclose nor suggest that aporphine alkaloids can be purified via silica gel column chromatography. As provided above, in the two methods disclosed, preparative layer chromatography was used rather than column chromatography. Boulware *et al.* did, however, use silica gel column chromatography to purify the alkaloids haplopine, hordenine and N-methyltyramine, which are not aporphine alkaloids. Contrary to the Examiner's assertion Applicant maintains that Boulware provides no motivation whatsoever to purify aporphine alkaloids via silica gel column chromatography. Applicant maintains therefore that the Boulware *et al.* reference does not render the method of this invention obvious. Applicant respectfully requests that this rejection be withdrawn.

Applicant believes that the pending claims are in condition for allowance. If it would be helpful to obtain favorable consideration of this case, the Examiner is encouraged to call and discuss this case with the undersigned. .

This constitutes a request for any needed extension of time and an authorization to charge all fees therefore to deposit account No. 19-5117, if not otherwise specifically requested. The undersigned hereby authorizes the charge of any fees created by the filing of this document or any deficiency of fees submitted herewith to be charged to deposit account No. 19-5117.

Respectfully submitted,

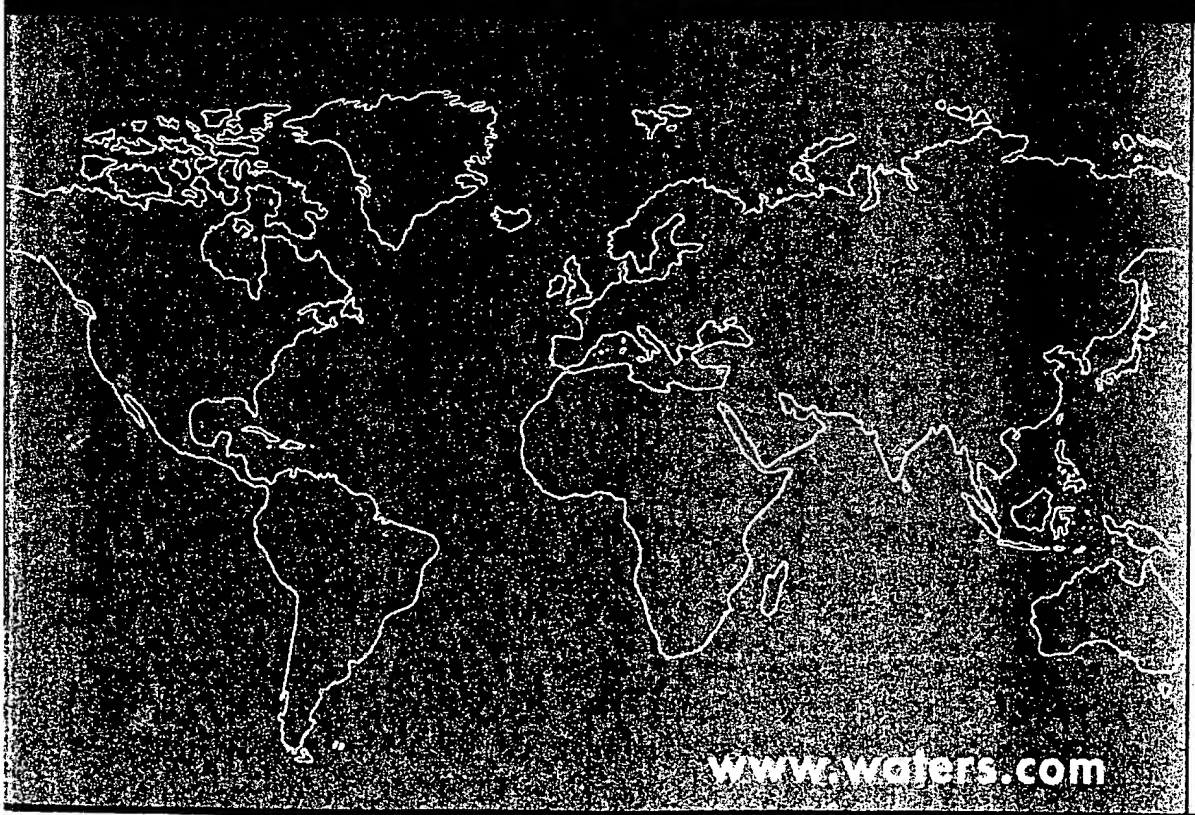
Date September 1, 2004

Rosemary P. Kellogg, #39,726
Swanson & Bratschun, L.L.C.
1745 Shea Center Drive, Suite 330
Highlands Ranch, Colorado 80129
Telephone: (303) 268-0066
Facsimile: (303) 268-0065

cc: UniGen Pharmaceuticals
S:\CLIENTFOLDERS\UNIGEN PHARMACEUTICALS\UNI15\DIV\UNI 15 D OA 6 9-1.DOC

# CHROMATOGRAPHY COLUMNS AND SUPPLIES CATALOG






2003 – 2004

## Specific Pore Volume and Particle Strength

The specific pore volume is a measure of the empty space in a particle. The larger the specific pore volume, the smaller is the fraction of the particle that is occupied by the particle skeleton. Therefore, particles with a large specific pore volume are less strong than particles with a small specific pore volume. Silicas with a specific pore volume of 0.5 mL/g or less, such as Waters Spherisorb® or Nova-Pak®, are mechanically extremely rugged. Many HPLC-grade silicas have a specific pore volume of 1 mL/g, which is generally sufficient for most applications.

### Choosing a Rugged Packing

| Specific Pore Volume | Strength/Ruggedness |
|---|---|
| 1.0 mL/g | ++ |
| 0.5 mL/g | ++++ |

++++ Excellent ++ Average

### Specific Pore Volume and Size Exclusion Chromatography

In size-exclusion chromatography, the separation results from smaller size analytes traveling into and out of pores. They take more time to elute than larger size analytes that are too big to fit into the pores. The larger size analytes elute before the smaller size analytes. Therefore, particle porosity and pore size is one of the key parameters that determines the usefulness of a packing. The particle porosity increases with increasing specific pore volume. If silica-based packings are used for size-exclusion chromatography, packings with a specific pore volume of about 1 mL/g or higher are preferred.

### Choice of Different Base Materials

| Product | Specific Surface Area ($m^2/g$) | Specific Pore Volume (mL/g) | Pore Size (Å) |
|---|---|---|---|
| Atlantis™ $dC_{18}$ | 330 | 1.00 | 100 |
| µBondapak™ | 330 | 1.00 | 125 |
| Delta-Pak™ | 300 | 1.00 | 100 |
| Delta-Pak™ 300 | 125 | 1.00 | 300 |
| Nova-Pak® | 120 | 0.30 | 60 |
| Resolve™ | 200 | 0.50 | 90 |
| Symmetry® | 335 | 0.90 | 100 |
| Waters Spherisorb® | 200 | 0.50 | 80 |
| XTerra® | 175 | 0.70 | 125 |

### Comparison

| | Pore Size | Specific Pore Volume |
|---|---|---|
| Retentivity | small | — |
| Capacity | small | — |
| Strength | small | small |
| Mass Transfer | large | — |
| Size Exclusion | — | large |
| Large Analytes | large | — |

For retentivity, capacity and strength, packings with a small pore size should be selected. The lower limit is determined by mass transfer in the pores. For analytes with a larger molecular weight, packings with a larger pore size should be selected. For size-exclusion chromatography, packings with a large specific pore volume should be chosen.

### Surface Modified Base Materials — Bonded Phase and Mode of Separation

Most analytical chromatography is carried out using packing materials whose sorption properties have been modified by attaching a covalently bonded phase (ligand) to the surface of silica. Alternatively, the surface of a packing can be modified by coating it with a chemically stable adsorptive layer.

Chemically stable bonds between the packing and the ligands that are responsible for retention and selectivity are only available for silica and polymeric packings.

The surface of the silica can easily be derivatized through silanization (bonding). The most commonly used HPLC packing is obtained by derivatization of the surface of a silica sorbent with a long-chain aliphatic silane reagent (chemically reacts at the silanol site). The aliphatic chain is 18 carbons long, and the packing is called $C_{18}$ or ODS (for octadecyl silane).

Several other surface derivatizations (different ligands) of silica are available which result in packings with widely varying properties, especially selectivity. Also, polymeric packings with a similar range of surface properties are used in HPLC.

In the following, we will briefly discuss the available packings based on the mode of separation.

## Normal Phase Chromatography

Normal-phase chromatography is the classical form of chromatography using polar stationary phases and non-polar mobile phases. The solute is retained by the interaction of its polar functional groups with the polar groups on the surface of the packing. Classically unbonded silica and alumina have been used for this application, but today polar bonded phases can be used with the following advantages: bonded phases equilibrate faster, are less sensitive to minute concentrations of water in the mobile phase, and yield different selectivities.

### Choosing a Normal Phase Packing

| Packing | Application |
|---|---|
| Silica | general purpose sorbent |
| Alumina | general purpose sorbent, more retentive than silica; group separation of aromatic hydrocarbons |
| Diol | less retentive than silica, equilibrates quickly |
| CN | least retentive normal phase sorbent |
| $NH_2$ | most polar bonded phase; different selectivity than silica; group separation of aromatic hydrocarbons |

## Reversed-Phase Chromatography

Reversed-phase chromatography has become the most popular mode of chromatography. In reversed-phase chromatography, the stationary phase is non-polar and the mobile phase is polar. Typical mobile phases are mixtures of water or aqueous buffer with methanol, acetonitrile or tetrahydrofuran. Typical stationary phases are silica-based bonded phases with aliphatic hydrocarbons as ligands. Other packings for reversed-phase chromatography are graphitized carbon and styrene-divinylbenzene packings.

The performance of reversed-phase bonded phases depends also on the activity of residual (unbonded) silanols on the surface of the bonded particle (Si-OH). Silanols interact with the polar functional group of the solutes. Therefore, packings exhibit different selectivities depending on the activity of the silanols. Also, tailing peaks are often observed for basic compounds on packings with a high level of silanol activity. This tailing results from cation-exchange interaction with positively charged basic analytes when the silanol group becomes de-protonated at pH values > 3.

One way of modifying silanol activity is by endcapping, that is reaction with a silanization reagent that converts the silanols to trimethylsilyl groups. Nevertheless, the surface concentration of residual silanols is always higher than the total concentration of bonded ligand including the endcapping ligand. Silanol activity also depends on the pretreatment of the silica ("base-deactivation") and the purity of the silica (contamination of base material from metals, such as aluminum). Fully endcapped bonded phases based on high-purity silicas are recommended for the chromatography of basic analytes. Non-endcapped packings can be used with advantage in many other applications to obtain a different selectivity.

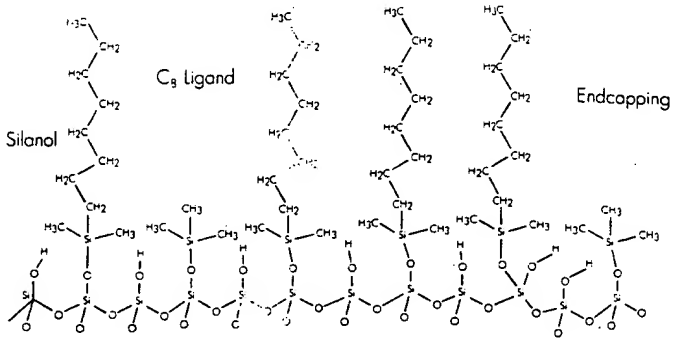
### Reversed-Phase Packings

Common reversed-phase packings include:

| | | |
|---|---|---|
| $C_{18}$ (ODS) | $C_4$ (Butyl) | CN (Nitrile) (Cyano) |
| $C_8$ (Octyl) (MOS) ($RP_8$) | $C_3$ (Propyl) | Shielded Reversed-Phase |
| $C_6$ (Hexyl) | $C_2$ (Ethyl) ($RP_2$) | Polymer RP |
| P (Phenyl) | $C_1$ (Methyl) (SAS) | |

Diagrams of these ligands are shown on page 98.

### Surface of a Typical Reversed-Phase Packing




## Ion-Exchange Chromatography

The separation of solutes by their charge is possible by using phases which contain fixed ionic charges. In the case of silica-based ion-exchangers, the ionic species are attached to the surface using standard silanization techniques. In the case of polymer-based ion-exchangers, the ion-exchange groups are distributed throughout the matrix. There are four categories of ion-exchangers: strong and weak cation exchangers and strong and weak anion exchangers. Weak ion-exchangers are characterized by the fact that the charge on the packing material is a function of the pH. Exchangers with carboxylic acids as functional groups are an example of weak cation exchangers. Weak anion exchangers comprise primary, secondary and tertiary amines.

The charge of strong ion-exchangers is for the most part independent of pH. Quaternary amines form strong anion exchangers, and sulfonic acids are classified as strong cation exchangers. All these functional groups are available on polymeric packings, primarily for the separation of large biomolecules. All but the weak cation exchanger are available on silica. Also, special ion-exchangers are available for ion chromatography. The latter are characterized by a low ion-exchange capacity, which makes it possible to use them with low-ionic-strength mobile phase, which is a requirement for ion chromatography with conductivity detection.

### Designations of Ion-Exchangers

| Strong Anion Exchanger | Weak Anion Exchangers | Strong Cation Exchangers | Weak Cation Exchangers |
|---|---|---|---|
| SAX | $NH_2$ | SCX | CM |
| | | DEAE | |

## Ion-Suppression Chromatography

This technique is widely used for the analysis of organic acids in eluents at low pH. It is simply a subcategory of reversed-phase chromatography. Special polymer phases are available for this application. In addition silica phases with $C_{18}$, $C_8$ or $C_6$ ligands may also be used with pH 2 eluents.

## Hydrophilic-Interaction Chromatography

Hydrophilic-interaction chromatography is the extension of normal-phase chromatography to aqueous eluents. Polar stationary phases are used in conjunction with aqueous-organic mobile phases. Contrary to reversed-phase chromatography, retention increases with increasing organic content. The most popular stationary phase for this application is an aminopropyl bonded phase, however, native silica, diol phases or other polar phases can also be used. The most common application is the separation of carbohydrates using the aminopropyl phase. Columns specially prepared for this application are available under the name of carbohydrate columns.

### Hydrophilic Interaction Chromatography Packings

| | |
|---|---|
| $NH_2$ (Amino) | Alumina |
| Diol | Carbohydrate |
| Silica | Polar Polymeric Packings |

## The Nature of Bonded Phases

### Carbon Load and Ligand Density

### Measurement and Reporting

The carbon content of a packing material is determined by elemental analysis. Often the results are reported directly as the carbon load (weight % C) of a packing. From this value, and with the knowledge of the specific surface area of the packing and the molecular weight of the bonded ligand, another term, the ligand density can be calculated.

The ligand density is usually expressed in µmol/m², and indicates the concentration, or density of the ligands on the particle surface. It is also sometimes called the surface coverage. The ligand density is a very important measure of the composition of the surface of the bonded phase, and is one of the important parameters determining the selectivity of a packing material.

### Carbon Load

At equal ligand density on the same base silica, packings with larger ligands have a higher carbon load. In other words, $C_{18}$ phases have a higher carbon load than $C_1$ phases. For the same ligand on the same silica, the carbon load increases with surface coverage.

For the same ligand at equal surface coverage on packings with different pore sizes, the carbon load decreases with increasing pore size, due to decreasing specific surface area. From this discussion, we can see that carbon load alone is not a good measure for comparing the retentivity or the selectivity of two different brands of packing materials.

### Ligand Density

Ligand density, also called surface coverage, is a much better measure of the characteristic properties of a packing. It is calculated using the following equation:

$$\chi = \frac{\%C}{100 \cdot SA \cdot nC \cdot 12 \cdot \left(1 - \frac{\%C}{100} \cdot \frac{MW - 1}{nC \cdot 12}\right)} = moles/m^2$$

*SA* is the specific surface area, %C is the carbon load, *MW* is the molecular weight of the ligand and nC is the number of carbon atoms in the ligand molecule.

The higher this number, the higher is the density of the primary ligand on the surface of the packing. Since more ligands have been reacted at the silanol sites, this means that it also measures the reduction of the density of silanols on the surface (higher ligand densities means less residual silanol groups).

On a fully hydroxylated silica, the surface concentration of silanols is around 8 µmol/m². Ligand densities of the primary ligand vary between 0.5 to 4 µmol/m² for different bonded phases based on monofunctional ligands (see discussion below).

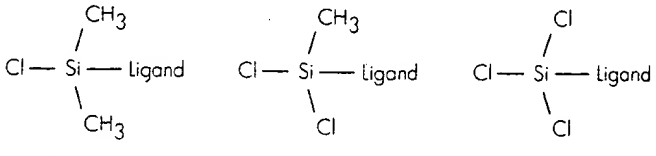
It is important to note that manufacturing reproducibility of the ligand density (tight specification) is of utmost importance for the reproducibility of the retentivity and selectivity of the final packing material in the HPLC column. In addition, packings with a high ligand density tend to be hydrolytically more stable than packings with a low ligand density at low pH mobile phase conditions.

### Some $C_{18}$ Packing Brands Grouped by Ligand Density

| Low | Medium | High |
|---|---|---|
| Atlantis™ $dC_{18}$ | XTerra® | Symmetry® $C_{18}$ |
| µBondapak™ $C_{18}$ | Resolve™ $C_{18}$ | Nova-Pak® $C_{18}$ |
| Waters Spherisorb® ODS1 | Waters Spherisorb® ODS2 | |

### Type of Bonded Phase and Endcapping

Bonded phases are produced by the chemical reaction of an organosilane reagent with the silica surface. The target is to achieve a uniform monomolecular layer. There are different types of silanes that are used for this bonding reaction. The silanes are characterized as mono-, di-, or trifunctional silanes depending on the number of groups on the silane (typically Cl groups) that can react with the silica surface.




**Monofunctional** **Difunctional** **Trifunctional**

With monofunctional silanes, the ligand density does not exceed 4 µmol/m² due to steric hindrance of the porous surface. Packings with a low ligand density are referred to as "monomeric". With multifunctional silanes, higher ligand densities can be achieved with some cross-linking between ligands. Often, packings with these higher ligand densities are referred to as "polymeric" packings, although there is little evidence for the formation of a polymer.

The selectivity of a packing depends on the ligand density. Especially different shape selectivities have been demonstrated for polyaromatic hydrocarbons at different ligand densities. The majority of commercially available bonded phases have a ligand density under 4 µmol/m² and are therefore of the monomeric type.

Monofunctional ligands give a more predictable coverage of the silica since complicating side reactions of the second reactive group are not possible. However, only one bond is made with the surface, which can result in an accelerated hydrolysis especially at low pH compared with phases prepared from multifunctional ligands. This hydrolysis breaks the ligand bond to the particle surface, and the ligand can then be washed from the column thereby changing its' chromatographic behavior.

Difunctional and trifunctional ligands are more difficult to bond reproducibly to the surface (batch –to- batch variability). On the average, two bonds are made with the surface, which increases the resistance to hydrolysis. Di- and trifunctional ligands are preferred when low pH mobile phases are used.

Manufacturers do not always reveal their bonding chemistries. However, most will indicate if a $C_{18}$ phase is monofunctional or not. More recently introduced reversed-phase packings are usually of that type because bonded phases can be produced with a higher reproducibility using monofunctional silanes.

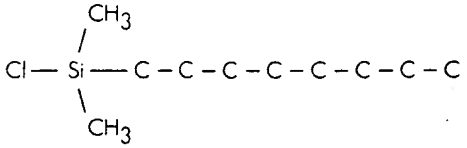
For applications in hydrolyzing conditions of high or low pH and high aqueous content, it is best to select a robust silica with difunctional or trifunctional bonding. All silica-based ion-exchangers are trifunctional. If nitrile (CN) packings are being used for reversed-phase chromatography, the better packings are based on a robust silica (low pore volume) and a trifunctional silane. Some manufacturers produce a nitrile bonding specifically for use in reversed-phase chromatography.

| Monofunctional | Difunctional | Trifunctional |
|---|---|---|
| Symmetry® $C_{18}$ | Atlantis™ $dC_{18}$ | Waters Spherisorb® ODS2 |
| Nova-Pak® $C_{18}$ | XTerra® Phenyl | Waters Spherisorb® CN |
| µBondapak™ $C_{18}$ | | XTerra® MS $C_{18}$ |
| XTerra® $RP_{18}$ | | XTerra® MS $C_8$ |
| XTerra® $RP_8$ | | |

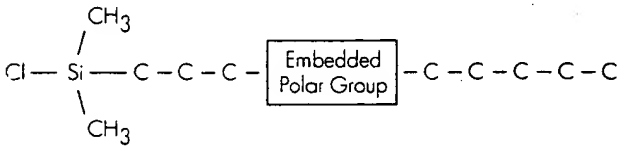
**Embedded Polar Group Ligands**

Advanced ligand technology now includes embedded polar groups as a part of the ligand chain. These provide several key benefits when compared to traditional, straight chain ligands:

- Best peak shape for basic analytes (Shields Silanols)
- Different Selectivity (powerful for methods development)
- Enhanced water wettability



**Straight Chain $C_8$**




**Embedded Polar $RP_8$**

**Embedded Polar Group Packings**

| | |
|---|---|
| SymmetryShield™ $RP_{18}$ | XTerra® $RP_{18}$ |
| SymmetryShield™ $RP_8$ | XTerra® $RP_8$ |

## Endcapping

For many reversed-phase packings, a secondary bonding step is carried out to cover unreacted silanol sites on the silica surface. A small silane, usually trimethylchlorosilane (TMCS), is used to produce a maximum coverage. This process is called endcapping. Endcapping is applied to most bonded phases used in reversed-phase chromatography. Phases used in normal-phase chromatography or other modes of chromatography are not endcapped. Reversed-phase packings that are not endcapped often exhibit a significantly different selectivity than endcapped packings. However, basic analytes tail on non-endcapped reversed-phase packings. Trimethylsilyl groups (endcapping groups) are especially subject to hydrolysis in acidic conditions. Therefore, endcapped packings should not be used at pH < 2.

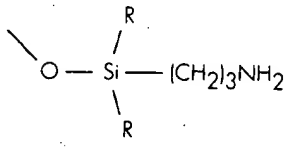
**Some Reversed-Phase Packings without Endcapping**

Waters Spherisorb® ODS1

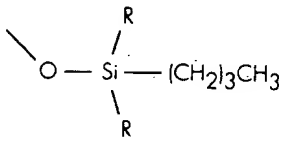
Resolve™ $C_{18}$ and $C_8$

# HPLC Silica Bonded*-Phase Structures

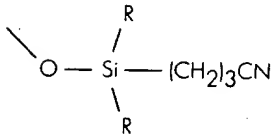
**Amino, $NH_2$**



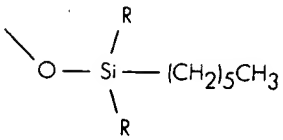
**Butyl, $C_4$**



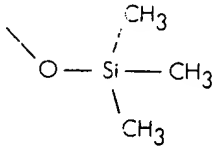
**Cyano, CN, Nitrile**



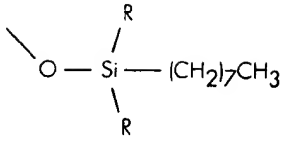
**Hexyl, $C_6$**



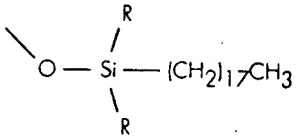
**Methyl, $C_1$**



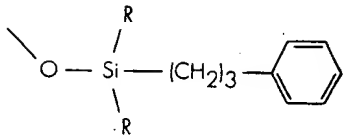
**Octyl, $C_8$**



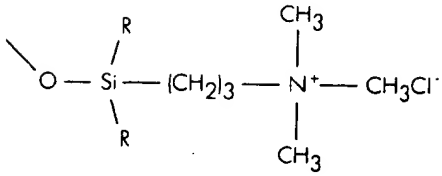
**ODS, $C_{18}$**



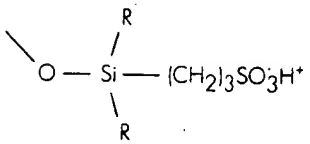
**Phenyl**



**SAX**



**SCX**



* Monofunctional where R is nonreactive.
Multi- or polyfunctional where R group is reactive.